REMARKS

Claims 1-3 are under examination in the present case. Claim 2 is rejected under 35 U.S.C. § 102. Claim 3 is rejected under 35 U.S.C. § 103(a). Claims 1 and 3 are rejected under 35 U.S.C. § 112, first paragraph. The rejections are addressed below.

Support for the Amendment

Support for the amendment of the claims is found throughout the specification as originally filed. For example, support for the amendment of claim 2, which now recites "normal longevity" is found at page 89, lines 6 and 7, and at page 114, lines 1-5.

Claim Election

Applicants affirm their election without traverse of Group I, claims 1-3.

Rejections under 35 U.S.C. § 102(b)

Claim 2 is rejected, under 35 U.S.C. § 102(b), as anticipated by Myers et al. (*PNAS* 94:9052-9057, 1997; hereafter "Myers"). This rejection is respectfully traversed.

Amended claim 2 features a method of diagnosing the normal longevity of a patient. The method involves analyzing the level of PTEN expression or activity in a sample isolated from a patient, where a decrease in the level of PTEN expression or activity relative to a control sample is an indication of decreased longevity. As indicated

in the specification, for example, at pages 113-114 and 117, PTEN plays a role in longevity, or normal lifespan, control. Nowhere is this claimed invention taught by the Myers reference.

A prior art reference anticipates a patent claim only if the reference discloses, either expressly or inherently, all of the limitations of the claim (*Continental Can Co. USA v. Monsanto Co.,* 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991)). The Office acknowledges, as is appropriate, that Myers does not expressly disclose a method for diagnosing longevity that involves measurement of PTEN expression or activity.

In rejecting claim 2 over Myers, the Office relies instead entirely on inherency, stating:

> The cited art further establishes a correlation between the severity in the disruption of PTEN activity and the pathology of diseases (The scientific study of the nature of disease and its causes processes, development, and consequences) like Bannayan-Zonana and Cowden disease (page 9057, col.1 para.3). Given the broadest reasonable interpretation the development of a cancer or tumors has inherently been associated with decreased longevity in a patient. Thus the cited art clearly anticipate the invention as claimed (Office Action, pages 5-6, emphasis added).

Applicants respectfully disagree.

First, Applicants point out that a diagnosis of a patient's "normal" lifespan differs from the likelihood that a patient will prematurely develop cancer, in this case due to the absence of PTEN tumor suppressor activity. The first depends, for example, on normal metabolic factors of the sort discussed throughout Applicants' specification; it reflects the

natural time-clock of the individual and is determined by their rate of aging. The second is entirely dependent upon the statistical likelihood that, at any given point in time, a given patient will develop cancer due to a mutation that increases their susceptibility; some individuals will develop the disease, others will not. The Office has incorrectly equated rate of aging with the statistical likelihood that a patient will develop a disease. The Myers reference – which is limited to a discussion of PTEN's role in disease susceptibility – cannot be anticipatory (either expressly or inherently) to a claim directed to the diagnosis of the rate of normal aging.

In addition, Myers fails to inherently anticipate the claimed method because it fails to satisfy the strict "certainty" standard set out by the case law for inherent anticipation. As summarized in the recent Federal Circuit decision, *Finnigan Corp. v. ITC*, 51 U.S.P.Q.2d 1001, 1009 (Fed. Cir. 1999) (citing *Continental Can Co., USA v. Monsanto Co.*, 948 F.2d 1264 (Fed. Cir. 1991)) (emphasis added):

> To serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence.. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. *In re Oelrich,* 666 F.2d 578, 581, 212USPQ 323, 326 (CCPA 1981)(quoting *Hansgirg v. Kemmer,* 102 F.d 212, 214, 40 USPQ 665, 667 (CCPA 1939)) [states]:
>
> Inherency, however may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.

While the Office states that "germ line mutation in PTEN gene give rise to

Cowden disease which is associated with the formation of multiple benign tumors and increases susceptibility to malignant cancers," such an increase in susceptibility to cancer is not sufficient to establish an anticipation rejection based on inherency because such mutations do not "necessarily" give rise to malignant cancers. The Examiner is directed, for example, to the accompanying medical summaries describing Bannayan-Zonana syndrome, discussed by Myers as one disorder associated with PTEN mutations. In the first of these publications (Appendix A), WebMD lists a number of characteristic outcomes of this disorder, none of which are fatal. In the second publication (Appendix B), The Doctor's Doctor echoes this list and specifically states that any concern with malignancies is due to the discovery that PTEN mutations are sometimes associated with Cowden's syndrome. As stated by the authors (emphasis added):

> Because of recently discovered genetic findings, there is evidence that Cowden's syndrome and this disease share a common genetic abnormality. Prior to this discovery, there was no increased risk of malignancy in kindreds. However, in light of this new discovery, it has been suggested that there <u>may</u> be an increased risk of malignancy.

Accordingly, far from being "necessarily" true that a PTEN mutation results in the development of a malignancy, it instead is clear that patients – even those known to have PTEN mutations – are only possibly at an increased cancer risk. This does not satisfy the standard for inherency. As stated above by the Federal Circuit, "inherency...<u>may not be established by probabilities or possibilities</u>. The mere fact that a certain thing <u>may</u> result from a given set of circumstances is not sufficient." Moreover, it is clear that disruptions

in this gene can lead to at least one disorder, Bannayan-Zonana syndrome, which, although characterized by the development of benign lesions, does not appear to be associated with an increase in malignancies. Again, this highlights the fact that PTEN mutations are not "necessarily" associated with malignancies and do not "necessarily" result in a decrease in an individual's lifespan.

For all of the above reasons, the inherency rejection of claim 2 should be withdrawn.

Rejections under 35 U.S.C. § 103(a)

The Office rejects claim 3 under 35 U.S.C. § 103(a) over Myers et al. (*PNAS* 94:9052-9057, 1997) and Maehama et al. (*J. Biol. Chem. 237*:13375-13378, 1998, hereafter "Maehama").

Claim 3 is directed to a method for diagnosing longevity in a patient. The method involves analyzing the level of PTEN expression or activity in a sample isolated from a patient by detecting PTEN lipid phosphatase activity, where a decrease in the level of PTEN expression or activity relative to a control sample is an indication of decreased longevity.

The Office states that "[I]t would have been obvious to one of ordinary skill in the art at the time of filing to modify the method as taught by Myers by substituting the substrate of PTEN with PtdIns(3,4,5)P_3." This rejection is respectfully traversed.

As indicated above, Myers fails to teach or suggest a role for PTEN in longevity. Maehama fails to remedy this deficiency. Maehama describes the overexpression of wild-type and mutant PTEN polypeptides. Like Myers, Maehama fails to teach or suggest that PTEN functions in mammalian longevity. Rather, Maehama merely characterizes the enzymatic activity of recombinant PTEN. Thus, even when the Office combines the teachings of Myers with the teachings of Maehama, it does not arrive at the claimed diagnostic method. The § 103 rejection as applied to this claim should be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 1 and 3, which are directed to methods for diagnosing an impaired glucose tolerance condition or obesity in a patient, are also rejected as lacking enablement. This enablement rejection turns on the following assertions: (i) that Applicants have failed to establish a role for PTEN in mammalian glucose homeostasis; (ii) that the state of the art relating to impaired glucose tolerance and obesity conditions is unpredictable, given that the development of the conditions is complex and multi-factorial; and (iii) that Applicants have failed to provide sufficient guidance regarding how to measure a decrease in PTEN levels as a means of diagnosing an impaired glucose tolerance condition. This rejection is respectfully traversed.

Role of PTEN in mammalian glucose homeostasis

Contrary to the Office's assertion that Applicants have failed to establish a role for PTEN in mammalian glucose homeostasis, Applicants clearly disclose that PTEN modulates mammalian insulin signaling, just as *C. elegans* Daf-18 modulates insulin signaling in the worm (page 117, line 1, to line 8). Applicants state:

> Reduction in PTEN activity would be expected to potentiate insulin and/or insulin-like growth factor signaling, but an increase of PTEN activity would be expected to cause insulin resistance downstream of the insulin receptor, the type observed in late onset diabetes. (page 117, lines 3-7.)

In addition, Applicants disclose that their results regarding the regulation of insulin signaling in *C. elegans* are relevant to mammalian insulin signaling.

> These results further endorse the congruence between the *C. elegans* and mammalian insulin signaling pathways, strongly supporting the contention that new genes identified in the *C. elegans* pathway also act in mammalian insulin signaling. In addition, we have also found that the *C. elegans* PTEN lipid phosphatase homologue, DAF-18, acts upstream of AKT in this signaling pathway. Thus, our molecular genetic analysis maps mammalian PTEN action to the insulin signaling pathway. (page 3, lines 7-15.)

In addition, contrary to the Office's assertion that the specification discloses that limited homology exists between *C. elegans* and human PTEN, Applicants state:

> The sequence of a full length *daf-18* cDNA predicts a protein of 962 amino acids (Figures 40A and 40B). Homology between DAF-18 and human PTEN (U93051; Li et al. (1997) Science 275:1943-1947) is highest within the phosphatase domain (38% identical, 94/250 aa) which is located at the amino-terminal end of both proteins (Figures 39A and 39B). Amino acids surrounding the probable active site Cys-$(X)_5$-Arg sequence are 90% identical (18/20 aa) between DAF-18 and PTEN (Figure 39B). This suggests that the substrate specificity of DAF-18 and PTEN may be similar.

In sum, it is clear from the above that Applicants disclose that significant parallels exist

between *C. elegans* and mammalian insulin signaling, at the functional and structural levels, and that these parallels elucidate the role of PTEN in mammalian glucose homeostasis.

In addition, as further evidence that PTEN functions in mammalian glucose homeostasis, just as Applicants disclosed, Applicants provide Butler et al. (*Diabetes* 51:1028-1034, 2002; hereafter "Butler," Exhibit C). Butler teaches that inhibiting PTEN expression reverses hyperglycemia in diabetic (*db/db* and *ob/ob*) mice. Regarding the role of PTEN in regulating insulin signaling, Butler states:

> Systemic administration of PTEN ASO [antisense oligonucleotides] once a week in mice suppressed PTEN mRNA and protein expression in liver and fat by up to 90 and 75% respectively, and normalized blood glucose concentrations in *db/db* and *ob/ob* mice. Inhibition of PTEN expression also dramatically reduced insulin concentrations in *ob/ob* mice, improved the performance of *db/db* mice during insulin tolerance tests, and increased Akt phosphorylation in liver in response to insulin. These results suggest that PTEN plays a significant role in regulating glucose metabolism in vivo by negatively regulating insulin signaling. (page 1028, left column, Abstract, emphasis added.)

Therefore, as further evidenced by Butler, PTEN modulates mammalian insulin signaling, just as Applicants disclosed. This first basis for the enablement rejection should be withdrawn.

Unpredictability of impaired glucose tolerance and obesity

The second basis on which the enablement rejection turns is the Office's assertion that the state of the art relating to impaired glucose tolerance and obesity conditions is

unpredictable, given that the development of the conditions is complex and multi-factorial. Given this unpredictability, the Office further asserts that Applicants have failed to establish a role for PTEN in glucose homeostasis. In support of this rejection, the Office cites Hirosumi et al. (*Nature* 420:333-336, 2002; hereafter "Hirosumi"), Shulman (*Clin. Invest.* 106:171-176, 2000; hereafter "Shulman"), Lonnqvist et al. (*Nat. Med.* 1:950-953, 1995; hereafter "Lonnqvist"), Kahn et al. (*J. Clin. Invest.* 106:473-481, 2000; hereafter "Kahn"), and Fontaine (*JAMA* 289:187-193, 2003; hereafter "Fontaine"). These publications, however, do not call into question either Applicants' assertion that PTEN plays a role in glucose metabolism or that decreased PTEN levels or activity may be used to diagnose impaired glucose tolerance conditions. Each publication is discussed below.

Hirosumi, Shulman, and Lonnqvist

Hirosumi describes a role for c-Jun amino-terminal kinases in obesity and insulin resistance. Shulman provides a review of the cellular mechanisms that contribute to insulin resistance. Lonnqvist describes the overexpression of the obese (ob) gene in adipose tissue of obese human subjects. Hirosumi, Shulman, and Lonnqvist uniformly fail to address the role of PTEN in mammalian glucose metabolism and fail to address the possible use of PTEN level or activity in diagnosing an impaired glucose tolerance condition. Given this failure, the cited references cannot support the Office's rejection of the claims as lacking enablement.

Kahn

Kahn provides a review of the role of insulin resistance in diabetes and the relationship of obesity to insulin resistance and type 2 diabetes. Kahn teaches that adipose tissue can function as an endocrine organ releasing hormones that regulate energy balance and glucose homeostasis. Rather than supporting the Office's position, Kahn provides evidence that *C. elegans* discoveries in this area may be predictably transferred to human glucose metabolic processes. In particular, despite the multiplicity of factors that contribute to metabolism regulation, Kahn recognizes that essential aspects of insulin signaling are conserved from *C. elegans* to humans. Kahn states:

> Insulin's metabolic effects are mediated by a broad array of tissue-specific actions that involve rapid changes in protein phosphorylation and function, as well as changes in gene expression. The fundamental biologic importance of these actions of insulin is evidenced by the fact that the insulin signaling cascade which initiates these events is largely conserved in evolution from C. elegans to humans. (page 473, left column, second paragraph.)

Thus, Kahn, a reference cited by the Office, provides support for Applicants' disclosure that regulation of the *C. elegans* insulin pathway parallels the regulation of mammalian insulin signaling.

Fontaine

Fontaine describes differences in life expectancy observed in obese versus normal human subjects.

Regarding Fontaine, the Office states:

Obesity appears to lessen life expectance markedly especially among young adults

> (Fontaine et al JAMA 289:1887-193, 2003). Therefore considering the Applicant's disclosure it is even unclear how one skill in the art would conclude that increase in PTEN activity that causes obesity would not leads to decreased longevity or visa versa [sic] (especially in context of invention as claimed in claim 2).

The Office appears to be concerned that the claimed methods of diagnosing obesity and longevity are contradictory, given that Applicants disclose that an increase in the level of PTEN expression or activity relative to a control sample is an indication of an impaired glucose tolerance condition or obesity, while a decrease in the level of PTEN expression or activity relative to a control sample is an indication of decreased longevity. As detailed below, this concern is misplaced.

It does not logically follow, nor do Applicants teach, that *any* decrease in the level of PTEN would necessarily result in a decrease in longevity. Rather, applicants' claimed diagnostic methods require that decreases in PTEN levels are *relative to a control sample* and increases in PTEN expression or activity are *relative to a control sample*. Based on the teaching of Applicants' specification, methods that decrease *elevated* PTEN levels would be expected to treat obesity. Given that obesity has a devastating impact on human health and life expectancy, as the Fontaine reference describes, the skilled artisan reading Applicants' disclosure understands that such methods would likely improve human health and increase longevity. Thus, this basis for the enablement rejection should be withdrawn.

Guidance regarding diagnostic methods

The third basis for the enablement rejection is the Office's assertion that Applicants have failed to provide sufficient guidance regarding how to carry out the claimed diagnostic methods. On this point, the Office first states:

> [C]onsidering the unpredictability in the art and the limited guidance provided in the specification as filed one skill in the art would have to engage in excessive and undue amount of experimentation to exercise the invention as claimed. The undue experimentation required would include scientific evaluation of the role of PTEN in impaired glucose tolerance and obesity especially in context with the multi factorial nature of these disorders. (page 12, line 18, to page 13, line 2, Office Action mailed August 21, 2003.)

Applicants disagree. As detailed above, Applicants have clearly disclosed and a reference cited by the Office (e.g., Kahn) accepts that mammalian PTEN functions in insulin signaling and metabolism. Thus, this basis for the rejection should be withdrawn.

In addition, the Office asserts that Applicants have not provided "reasonable detail" for carrying out the claimed diagnostic methods. Applicants respectfully disagree. Contrary to the Office's assertions, Applicants have provided a teaching in the present specification that enables the skilled artisan to practice the methods of the invention. Applicants teach methods for diagnosing an impaired glucose tolerance condition or obesity by analyzing the level of PTEN expression or activity in a sample isolated from the patient, where an increase in the level of PTEN expression or activity relative to a control sample is an indication of an impaired glucose tolerance condition or obesity. For example, Applicants teach that increases in PTEN levels or activity are expected to cause

insulin resistance downstream of the insulin receptor (page 117, lines 3-7). Applicants also teach that such resistance is associated with late onset diabetes (page 117, lines 3-7).

Methods for analyzing the level of PTEN activity are described at page 119, lines 2-5, where Applicants teach that DAF-18/PTEN activity may be identified using an *in vitro* lipid phosphatase assay as described by Maehama and Dixon (J. Biol. Chem. 273:13375, 1998). Methods for analyzing the level of PTEN expression are described, for example, at pages 150-152, where Applicants teach that anti-DAF polypeptide antibodies are useful in immunological assays to detect DAF polypeptide expression in a patient sample; at pages 176 and 177, where Applicants teach that a DAF promoter may be fused to a reporter and used to monitor DAF expression; and at page 195, lines 10-25, where Applicants teach methods of analyzing the expression of DAF-18 nucleic acid and amino acid sequences. In addition, Applicants teach that the appropriate controls for studies of insulin regulation are wild-type control mammals not having diabetes (page 126, line 10-12; page 200, lines 21-25; and page 202, lines 11-14).

In view of these teachings in the specification, it is clear that Applicants have provided an enabling description that would instruct the skilled artisan how to diagnose a glucose intolerance condition or obesity by analyzing DAF-18 expression. Accordingly, the enablement rejection should be withdrawn.

CONCLUSION

Applicants submit that this case is in condition for allowance, and such action is respectfully requested. Enclosed is a petition for extending the period for reply for two months, to and including, January 21, 2004. If there are any charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 21 January 2004

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Exhibit A

Bannayan-Zonana Syndrome

Background

This syndrome is very closely related to Cowden's syndrome with multiple hamartomas. Individuals with this syndrome frequently present with macrocephaly with developmental delay and hypotonia, which are recognized during the first few years of life. Hamartomatous growths such as intestinal polyposis, subcutaneous and visceral lipomas, and vascular malformations are common findings. Skin findings include pigmented macules of the penis.

Because of recently discovered genetic findings, there is evidence that Cowden's syndrome and this disease share a common genetic abnormality. Prior to this discovery, there was no increased risk of malignancy in kindreds. However, in light of this new discovery, it has been suggested that there may be an increased risk of malignancy.

SYNONYMS	Riley-Smith syndrome (1960) Bannayan-Zonana syndrome (1971) Ruvalcaba-Myre syndrome (1980) Bannayan-Riley-Ruvalcaba syndrome

DISEASE ASSOCIATIONS	**CHARACTERIZATION**
Bannayan-Zonana syndrome associated with lymphangiomyomatous lesions. **Klein JA, Barr RJ.** **Department of Dermatology, California College of Medicine, University of California-**	Pediatr Dermatol 1990 Mar;7(1):48-53 Abstract quote Bannayan-Zonana syndrome is an autosomal dominant condition that has not been well described in the dermatology literature. The typical case is characterized by macrocephaly, multiple angiomas, and multiple encapsulated or infiltrating lipomas. As in other autosomal dominant hamartoneoplastic syndromes, the degree of expression within one family frequently varies widely. Our patient had macrocephaly and angiomas, as well as lipomas with peculiar histologic features similar to lymphangiomyomas. Her father had a

Irvine.	large nevus flameus on his leg, and lipomas with normal histologic appearance. The paternal grandfather had multiple encapsulated lipomas with normal histologic appearance. Neither father nor grandfather had macrocephaly.

PATHOGENESIS	CHARACTERIZATION
Identification of a PTEN mutation in a family with Cowden syndrome and Bannayan-Zonana syndrome	J Am Acad Dermatol 2001;44:183-7 Genetic basis of these two diseases is identical. The PTEN gene (phosphatase and tensin homolog deleted from chromosome 10) has been identified as the susceptibility gene for CS and BZS, and thus mutations in PTEN have been reported in both syndromes PTEN, also known as MMAC1 (mutated in multiple advanced cancers) or TEP1 (TGF-regulated and e pithelial cell-enriched p hosphatase), is a tumor suppressor gene located on chromosome 10q23.28-30 It has 9 exons that encode a protein of 403 amino acids

Pediatrics 1982;69:632-4.
Am J Med Genet 1983;15:491-5.
Am J Med Genet 1984;19:225-34.
Arch Dermatol 1996;132:1214-8.
J Am Acad Dermatol 2001;44:183-7

Commonly Used Terms

Skin

Skin syndromes

TOP

Last Updated 2/18/2001

Send mail to psdermpath@earthlink.net with questions or comments about this web site.

Exhibit B

Health Guide A-Z

Bannayan Riley Ruvalcaba Syndrome

healthwise

Important

It is possible that the main title of the report Bannayan Riley Ruvalcaba Syndrome is not the name you expected. Please check the synonyms listing to find the alternate name(s) and disorder subdivision(s) covered by this report.

Synonyms

- BRRS
- Bannayan-Zonana syndrome (BZS)
- Riley-Smith syndrome
- Ruvalcaba-Myhre-Smith syndrome (RMSS)
- Macrocephaly, multiple lipomas, and hemangiomata
- Macrocephaly, pseudopapilledema, and multiple hemangiomata

Disorder Subdivisions

- None

General Discussion

Bannayan-Riley-Ruvalcaba syndrome is a rare inherited disorder characterized by excessive growth before and after birth; an abnormally large head (macrocephaly) that is often long and narrow (scaphocephaly); normal intelligence or mild mental retardation; and/or benign tumor-like growths (hamartomas) that, in most cases, occur below the surface of the skin (subcutaneously). The symptoms of this disorder vary greatly from case to case.

In most cases, infants with Bannayan-Riley-Ruvalcaba syndrome exhibit increased birth weight and length. As affected infants age, the growth rate slows and adults with this disorder often attain a height that is within the normal range. Additional findings associated with Bannayan-Riley-Ruvalcaba syndrome may include eye (ocular) abnormalities such as crossed eyes (strabismus), widely spaced eyes (ocular hypertelorism), deviation of one eye away from the other (exotropia), and/or abnormal elevation of the optic disc so that it appears swollen (pseudopapilledema). In addition, affected infants may also have diminished muscle tone (hypotonia); excessive drooling; delayed speech development; and/or a significant delay in the attainment of developmental milestones such as the ability to sit, stand, walk, etc. In some cases, multiple growths (hamartomatous polyps) may develop within the intestines (intestinal polyposis) and, in rare cases, the back wall of the throat (pharynx) and/or tonsils. Additional abnormalities associated with this disorder may include abnormal skin coloration (pigmentation) such as areas of skin that may appear "marbled" (cutis marmorata) and/or the development of freckle-like spots (pigmented macules) on the penis in males or the vulva in females. In some cases, affected individuals may also have skeletal abnormalities and/or abnormalities affecting the muscles (myopathy). Bannayan-Riley-Ruvalcaba syndrome is inherited as an autosomal dominant genetic trait.

Bannayan-Riley-Ruvalcaba is the name used to denote the combination of three conditions formerly recognized as separate disorders. These disorders are Bannayan-Zonana syndrome, Riley-Smith syndrome, and Ruvalcaba-Myhre-Smith syndrome.

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Resources

March of Dimes Birth Defects Foundation

1275 Mamaroneck Avenue

White Plains, NY 10605

Tel: (914)428-7100

Fax: (914)997-4763

Tel: (888)663-4637

TDD: (914)997-4764

Email: Askus@marchofdimes.com

Internet: http://www.marchofdimes.com

The Arc (a national organization on mental retardation)

1010 Wayne Ave

Suite 650

Specific Inhibition of PTEN Expression Reverses Hyperglycemia in Diabetic Mice

Madeline Butler, Robert A. McKay, Ian J. Popoff, William A. Gaarde, Donna Witchell, Susan F. Murray, Nicholas M. Dean, Sanjay Bhanot, and Brett P. Monia

Signaling through the phosphatidylinositol 3′-kinase (PI3K) pathway is crucial for metabolic responses to insulin, and defects in PI3K signaling have been demonstrated in type 2 diabetes. PTEN (MMAC1) is a lipid/protein phosphatase that can negatively regulate the PI3K pathway by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate, but it is unclear whether PTEN is physiologically relevant to insulin signaling in vivo. We employed an antisense oligonucleotide (ASO) strategy in an effort to specifically inhibit the expression of PTEN. Transfection of cells in culture with ASO targeting PTEN reduced PTEN mRNA and protein levels and increased insulin-stimulated Akt phosphorylation in α-mouse liver-12 (AML12) cells. Systemic administration of PTEN ASO once a week in mice suppressed PTEN mRNA and protein expression in liver and fat by up to 90 and 75%, respectively, and normalized blood glucose concentrations in *db/db* and *ob/ob* mice. Inhibition of PTEN expression also dramatically reduced insulin concentrations in *ob/ob* mice, improved the performance of *db/db* mice during insulin tolerance tests, and increased Akt phosphorylation in liver in response to insulin. These results suggest that PTEN plays a significant role in regulating glucose metabolism in vivo by negatively regulating insulin signaling. *Diabetes* 51:1028–1034, 2002

Phosphatidylinositol 3′-kinase (PI3K) is a crucial signaling enzyme whose activity is regulated by a variety of biological stimuli, including insulin (1). PI3K is composed of two subunits: the p85 regulatory subunit, containing two Src homology-2 domains, and the p110 catalytic subunit (2–4). Binding of insulin to its receptor activates the insulin receptor tyrosine kinase, resulting in autophosphorylation and phosphorylation of several substrates, including insulin receptor substrate (IRS)-1 through -4. IRS then binds to the regulatory subunit of PI3K through its Src homology domains, and this interaction activates the catalytic unit. Activated PI3K phosphorylates the 3′-position of the ring in inositol phospholipids, generating phosphatidylinositol (3,4), diphosphate, and phosphatidylinositol (3,4,5)-triphosphate (PIP3). The lipid products of PI3K initiate phosphorylation and activation of Akt, which is believed to act as a downstream mediator of many of the metabolic effects of insulin (5,6). Thus, the expression of inactive PI3K mutants or chemical agents, such as wortmannin and LY294002 that interfere with PI3K activity, inhibit Akt phosphorylation, glucose uptake, and glycogen and lipid synthesis in vitro (7,8).

PTEN (MMAC1/TEP1) is a dual-specificity protein phosphatase involved in signal transduction and tumor suppression (9,10). PTEN also has phosphoinositide 3′-phosphatase activity and is therefore capable of suppressing PI3K signaling by dephosphorylating PIP3 (11,12). Mutations in PTEN have been associated with several human cancers, and mice heterozygous for the PTEN gene have a high incidence of spontaneous tumors (13,14). Tumor cells and fibroblasts deficient in PTEN have elevated levels of PIP3 and phosphorylated Akt/protein kinase B (PKB) and are resistant to many apoptotic stimuli (15,16).

Because many of the metabolic effects of insulin are mediated through activation of PI3K and the subsequent rise in intracellular PIP3 concentrations, inhibition of a negative regulator of this pathway may enhance insulin signaling. Although the tumor-suppressive functions of PTEN have been elucidated, its physiological role in glucose metabolism in vivo is largely unknown. Inhibition of the *daf-18* gene, a homolog of PTEN in *Caenorhabditis elegans*, can partially bypass the need for DAF-2, an insulin receptor-like molecule (17,18). PTEN overexpression in vitro inhibits glucose uptake and GLUT4 transport in 3T3L1 cells, whereas microinjection of PTEN antibodies increased GLUT4 translocation (19). These results suggest that PTEN may modulate insulin signaling in vivo; however, the lethality of the PTEN null mutation has made this difficult to study. We therefore designed and characterized antisense oligonucleotides (ASOs) targeting PTEN and used them in vitro and in vivo to determine whether the inhibition of PTEN expression affects insulin signaling and glucose metabolism.

RESEARCH DESIGN AND METHODS

Oligonucleotides. A total of 80 oligonucleotides were screened for their ability to inhibit PTEN mRNA expression in T-24 bladder carcinoma cells by quantitative real-time RT-PCR. All oligonucleotides were synthesized as uniform phosphorothioate chimeric oligonucleotides, with 2′-*O*-methoxyethyl groups on bases 1–5 and 16–20. The oligonucleotides were synthesized using an Applied Biosystems 380B automated DNA synthesizer (Perkin Elmer-Applied Biosystems) and purified as described (20). Two active PTEN ASOs complementary to human and mouse PTEN mRNA (Genbank accession nos. AA017584 and AA124728, respectively), a six-base mismatch, and a control

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AS1, antisense oligonucleotide 1; ASO, antisense oligonucleotide; DMEM, Dulbecco's modified Eagle's medium; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; IRS, insulin receptor substrate; MIS, mismatch control oligonucleotide; PI3K, phosphatidylinositol 3′-kinase; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PKB, protein kinase B; UC, universal control oligonucleotide.

oligonucleotide were used in the experiments described and are designated as follows: antisense oligonucleotide 1 (AS1) (ISIS 116847: 5′-CTGCTAGCCTCT GGATTTGA-3′, beginning at position 2097 in the human RNA); AS2 (ISIS 116845: 5′-CACATAGCGCCTCTGACTGGG-3′, beginning at position 1,539); MIS (ISIS 116848: 5′-CTTCTGGCATCCGGTTTAGA-3′, a six base mismatch to AS1); and UC, a universal control (ISIS 29848: synthesized using a mix of random mixture of A, G, T, and C so that the resulting preparation represents an equimolar mixture of all possible four [19] oligonucleotides).

Cell culture. 3T3L1 murine fibroblasts (American *Type Culture* Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (growth media). Cells were grown to confluence in 12- or 24-well plates before initiating differentiation. Confluent monolayers were differentiated to the adipocyte phenotype by culturing with 500 μmol/l isobutulmethylaxanthine, 250 nmol/l dexamethasone, and 400 nmol/l insulin in growth media for 3 days, followed by growth media alone for 3 days. Following this protocol, >90% of the adipocytes express the fully differentiated phenotype by 6 days after initiation.

Fully differentiated 3T3L1 adipocytes were transfected by the addition of serum-free DMEM and FuGENE6 (Roche) following the manufacturers instructions. The final concentration of 500 nmol/l oligonucleotide and a ratio of 4 μl FuGENE6 per microgram oligonucleotide were empirically determined to maximally suppress target RNA expression. Cell media was typically refreshed 36 h after transfection.

AML12 cells (American *Type Culture* Collection), a nontransformed hepatocyte cell line from transforming growth factor-β transgenic mice, were used to demonstrate antisense-mediated PTEN protein reduction and insulin-stimulated Akt phosphorylation in vitro. The cells were maintained in 90% of 1:1 mixture of DMEM and Ham's F12 medium containing 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, and 10% fetal bovine serum. The cells were treated with AS1 or mismatch control oligonucleotide (MIS) for 72 h using Lipofectin (Gibco) as a transfection agent per the manufacturer's instructions. Because AML12 cells require insulin for maintenance, the cells were serum- and insulin-starved for 8 h after transfection, and then 100 nmol/l insulin was added for 30 min before harvesting in lysis buffer for Western blotting.

Northern blots. RNA was prepared from cultured cells using a Qiagen RNA Easy Kit and from animal tissues homogenized in guanidinium isothiocyanate followed by cesium chloride gradients (21). Northern blots were performed as described using full cDNA probes generated by RT-PCR (22). The RNA signal was detected using a PhosphorImager (Molecular Dynamics) and normalized against the signal for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) using ImagePro software.

Western blots. Cells or tissues were harvested in lysis buffer (150 mmol/l NaCl, 50 mmol/l Tris, pH 7.5, 1% Triton X-100, 0.5% NP-40, 0.25% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.2 mmol/l ortho-vanadate, 1 mmol/l NaF, and 1:200 dilution of protease cocktail III; (Calbiochem), and the proteins were separated by SDS-PAGE. After transfer of the proteins onto polyvinylidine fluoride membranes, the blots were reacted with antibodies to phospho-Akt (New England Biolabs), Akt (New England Biolabs), or PTEN and developed using enhanced chemiluminescence (Amersham Pharmacia).

Mice and metabolic measurements. All animal experiments were performed under the institutional American Association for the Accreditation of Laboratory Animal Care (AALAC) guidelines. Male *db/db* mice (C57BLKS/J-*m* +/+*Lepr*^{db}) and age-matched lean littermates (C57BLKS/J-*m*+/+ *Lepr*^{db}) at 10 weeks of age or male *ob/ob* (C57BL/6J-*Lep*^{ob}) at 8 weeks of age (The Jackson Laboratory) were used for all experiments. Mice were maintained on a 12-h light/dark cycle and fed ad libitum unless otherwise noted. Whole blood was obtained from the retro-orbital sinus of fed mice, and glucose was measured using a Metabolics glucose oxidase-based analyzer. Mice were weighed once a week, and food intake was monitored over a 24-h period. An insulin tolerance test was performed after a 4-h fast by intraperitoneal injection of 1 unit/kg human insulin (Lilly). Blood was drawn from the tail before insulin injection (time 0) and then 30, 60, and 90 min afterward and measured as described above. For in vivo phospho-Akt measurements, mice were fasted for 12 h and then injected with 2 units/kg insulin.

Serum glucose, triglycerides, and cholesterol concentrations were analyzed on a Johnson and Johnson Vitros 950 automated clinical chemistry analyzer, and serum insulin concentrations were quantitated using an enzyme-linked immunosorbent assay for rat insulin (Alpco).

RESULTS

Characterization of PTEN ASO in vitro. ASOs designed to be complementary to human and mouse PTEN genomic sequences were screened for suppression of PTEN mRNA expression in cells in vitro, as previously described (23). The most potent oligonucleotide from the screen, AS1 (see RESEARCH DESIGN AND METHODS), reduced PTEN mRNA levels in a concentration-dependent manner in 3T3-L1 adipocytes. Similar results were obtained with a second PTEN antisense (AS2) that hybridizes to a different region of the PTEN mRNA. A control antisense with six mismatched nucleotides (MIS) to AS1 was inactive in reducing PTEN mRNA expression. Maximal inhibition (90%) of PTEN protein expression was achieved after 72 h of oligonucleotide treatment, which is presumably indicative of the intrinsic half-life of the PTEN protein (data not shown).

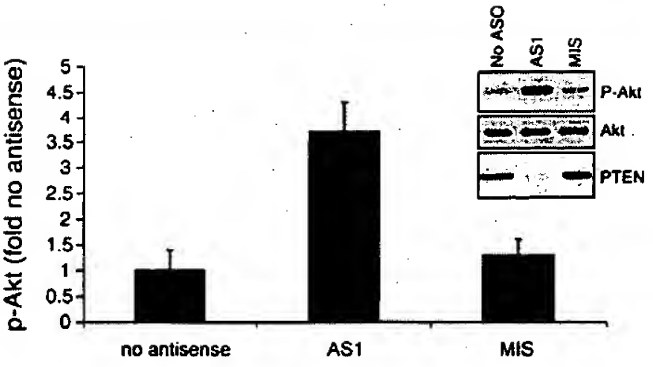

FIG. 1. Reduction of PTEN protein levels and stimulation of Akt phosphorylation by PTEN antisense treatment in vitro. AML12 cells were treated with AS1 or MIS for 72 h using Lipofectin as a transfection agent. Immunoblots of cell proteins were sequentially reacted with antibodies to phospho-Akt, -Akt, and -PTEN. Data are expressed as the fold increase in phospho-Akt intensity compared with Lipofectin-only treated cells. Graphs represent the mean of three replicates ±SD.

As described above, PIP3 is believed to initiate phosphorylation and activation of Akt, an important downstream mediator of the metabolic effects of insulin (24). We reasoned that if PTEN is indeed involved in modulating insulin signaling, inhibition of PTEN expression might increase the level of Akt phosphorylation in response to insulin. To test this, AML12 cells were treated with PTEN AS1, and the effects on PTEN protein levels and insulin-stimulated Akt phosphorylation were examined. Cells treated with PTEN AS1 resulted in a >90% reduction in PTEN protein levels (Fig. 1). Furthermore, PTEN AS1 treatment resulted in an increase in insulin-stimulated phosphorylation of Akt by ~3.5-fold, relative to untreated and MIS-treated cells (Fig. 1), whereas the Akt protein levels remained the same.

Antisense-mediated inhibition of PTEN expression in vivo. Based on the in vitro results obtained with AS1, we reasoned that inhibition of PTEN expression might improve insulin sensitivity in the *db/db* mouse, a rodent model of type 2 diabetes. First, we investigated the ability of systemically administered AS1 to reduce PTEN mRNA and protein levels in insulin-sensitive tissues. The half-life of 2′-*O*-methoxyethyl chimeric phosphorothioate oligonucleotides is ~7 to 19 days in the liver, depending on the dose (25). Therefore, *db/db* mice were treated by intraperitoneal injection once a week for 4 weeks with 10, 25, or 50 mg/kg of AS1, and PTEN mRNA levels in liver, fat, and muscle tissues were measured by Northern blotting. PTEN mRNA levels were reduced in a dose-dependent manner in

liver extracts from treated mice relative to saline controls, with maximal inhibition occurring (88%) at the 50-mg/kg dose (Fig. 2*A*). In lean littermates dosed with 100 mg/kg of AS1, PTEN mRNA levels were also reduced by >90%, relative to saline-treated controls. There was no apparent difference in the relative levels of PTEN mRNA in untreated lean versus *db/db* mice. Moreover, neither the MIS nor another control oligonucleotide, universal control oligonucleotide (UC), affected PTEN mRNA levels significantly. Also, the mRNA levels of PTP1B and SHIP2, two other phosphatases that have the potential to inhibit insulin signaling (26,27), were not affected by PTEN AS1 treatment (Fig. 2*B*). These results demonstrate that the effect of AS1 was both PTEN target–specific and antisense sequence–specific and indicate that the metabolic effects of the PTEN antisense (described below) were primarily caused by a specific reduction in PTEN expression.

PTEN protein levels in liver samples from saline-, AS1-, and UC-treated *db/db* mice were analyzed by Western blotting. After 4 weeks of AS1 treatment, a dose-dependent decrease in PTEN protein levels in livers from *db/db* mice was observed (Fig. 2*C*). Reduction of PTEN protein levels was also observed in livers from lean littermates treated with AS1. As with the mRNA results, no difference in the relative levels of PTEN protein in control lean versus *db/db* mouse livers was apparent, nor were any effects observed by a control oligonucleotide (UC) on PTEN protein levels.

Northern analysis of other insulin-sensitive tissues demonstrated that PTEN mRNA levels were also reduced in a dose-dependent manner, relative to saline controls, in fat tissue from AS1-treated *db/db* mice, with maximal inhibition of 80% at the 50 mg/kg dose (Fig. 2*D*). A similar reduction in PTEN protein levels in fat from AS1-treated mice was also observed (data not shown). PTEN message levels appeared to be less abundant in muscle relative to liver and fat, and no consistent reduction in PTEN mRNA expression was observed in the skeletal muscle of animals treated with PTEN AS1. This result is in agreement with pharmacokinetic studies showing that accumulation of oligonucleotides in muscle after parenteral injection is relatively low (28). Interestingly, no PTEN protein was detectable on immunoblots of muscle lysates (data not shown).

Effect of inhibiting PTEN expression on glucose, insulin, and lipid concentrations in diabetic and lean mice. Having characterized the effect of AS1 on PTEN mRNA and protein expression in vivo, we next investigated the effect of inhibiting PTEN expression on hyperglycemia in *db/db* and *ob/ob* mice. Blood glucose concentrations in *db/db* mice were reduced in a dose-dependent manner over the course of a 4-week treatment with AS1 (Fig. 3*A*), becoming normalized (138 ± 5 mg/dl) at the highest dose tested (50 mg/kg). The second PTEN antisense, AS2, produced a similar reduction in serum glucose levels at the end of 4 weeks of treatment (Fig. 3*B*). In related studies, treatment of *db/db* mice with PTEN AS2 resulted in a reduction of PTEN mRNA and protein levels in liver that was comparable with that produced in animals treated with PTEN AS2 (data not shown). In contrast, neither the MIS nor UC controls affected glucose levels. Furthermore, PTEN antisense treatment had no effect on glucose concentrations in lean littermates, despite the fact

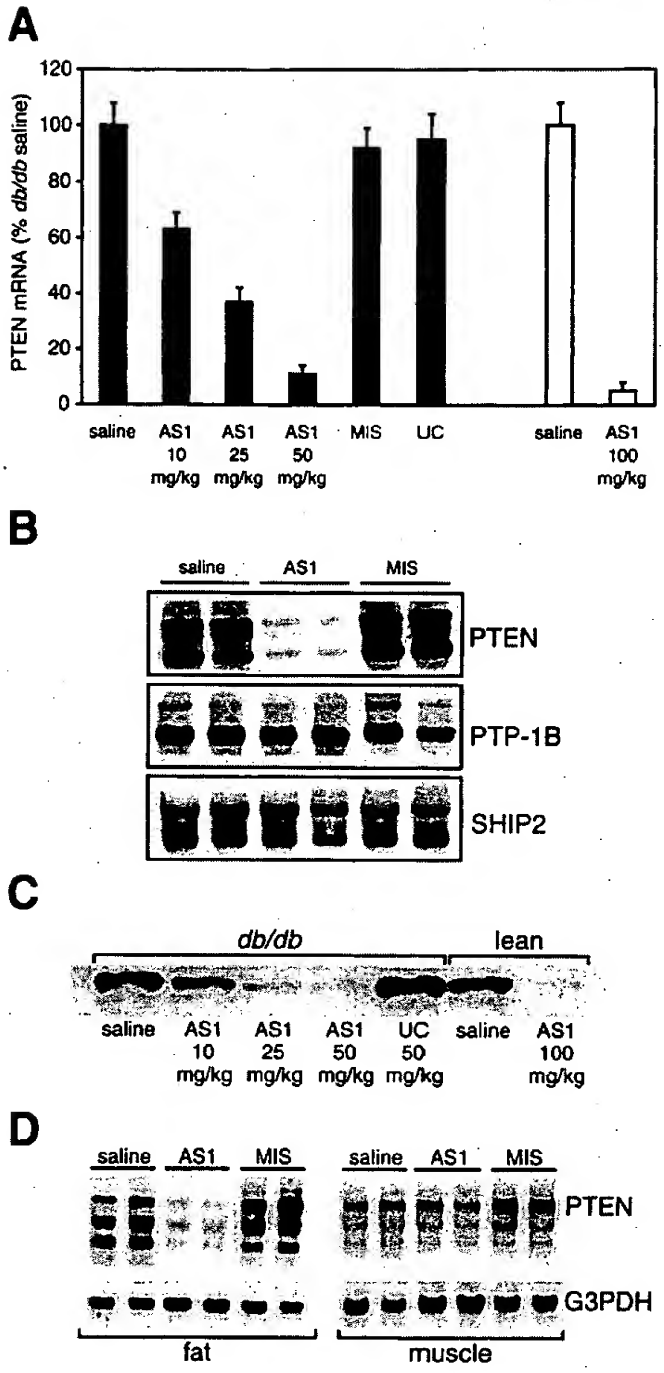

FIG. 2. PTEN antisense specifically reduces PTEN mRNA and protein levels in livers and fat from *db/db* mice. *A*: Dose-dependent reduction of PTEN mRNA levels in liver. *db/db* (black bars) and lean (white bars) mice received indicated doses of ASOs intraperitoneally once a week for 4 weeks. Total mRNA was prepared from liver and was analyzed by Northern blotting (n = 3 per group). The PTEN signal was normalized against the signal for G3PDH. Data are expressed as the mean percentage of mRNA levels in saline-treated *db/db* mice ±SD. *B*: Specificity of PTEN antisense. Representative Northern blots of PTEN mRNA (*A*), PTP1B mRNA (*B*), and SHIP2 mRNA (*C*) in livers from *db/db* mice treated once a week for 4 weeks with saline, 50 mg/kg AS1, or 50 mg/kg MIS. Each lane contained 25 μg of RNA from an individual animal. *C*: Reduction of PTEN protein expression in liver. PTEN immunoblots of proteins in liver lysates from mice treated for 4 weeks with indicated doses of AS1 or UC. Each lane contained 50 μg of protein. *D*: Reduction of PTEN mRNA in fat but not muscle. Representative Northern blots of PTEN and G3PDH mRNA in fat and muscle from *db/db* mice treated with saline, 50 mg/kg AS1, or 50 mg/kg MIS.

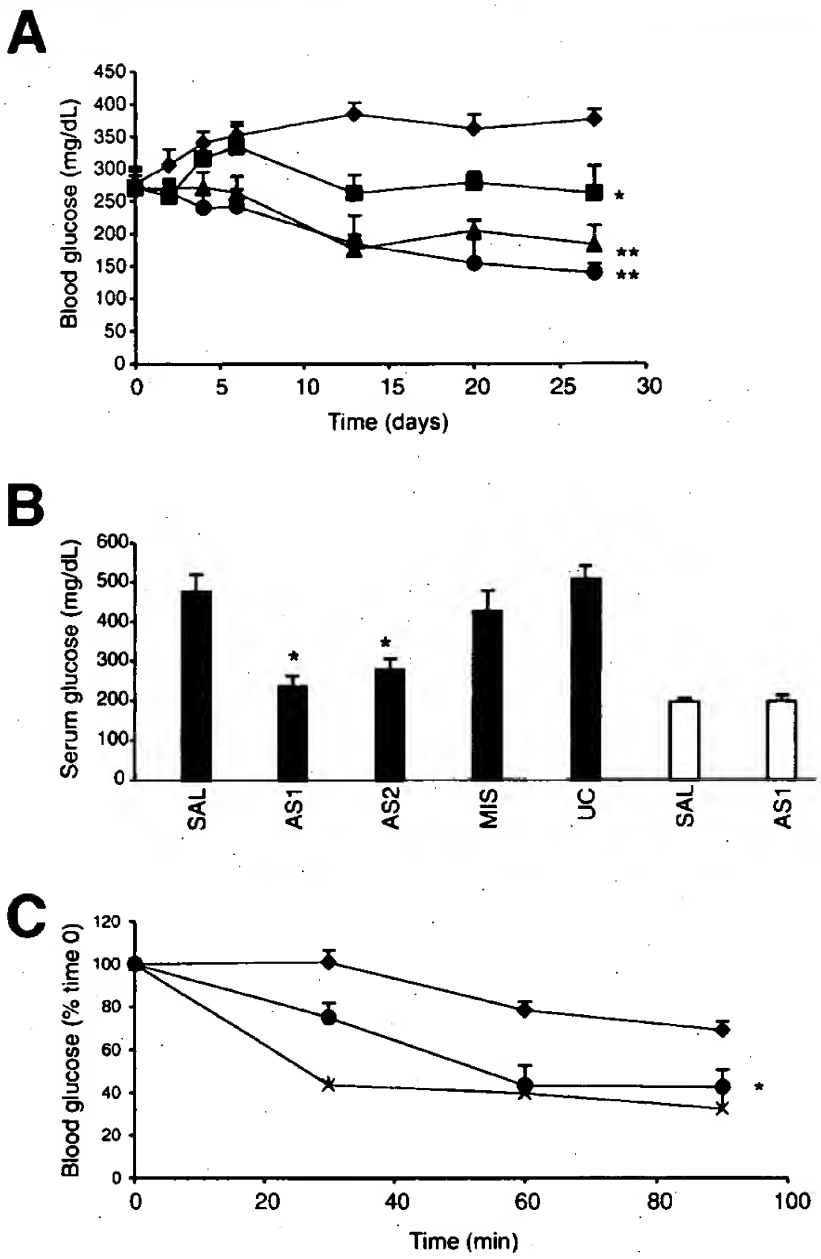

FIG. 3. Inhibition of PTEN expression lowers glucose and increases insulin sensitivity in *db/db* mice. *A*: Time- and dose-dependence of glucose-lowering effects of PTEN AS1. *db/db* mice were injected intraperitoneally once a week with saline (♦) or 10 mg/kg (■), 25 mg/kg (▲), or 50 mg/kg(●) of AS1 in saline. Mice were bled every 2 days for the first week and then once a week thereafter, 6 days after the previous dose. Values are expressed as means ± SE (n = 6–8). Statistical analysis was done using ANOVA repeated measures followed by Bonferroni-Dunn. Compared with saline-treated, **P < 0.0001, *P < 0.01. *B*: Specificity of the antidiabetic effect of PTEN ASOs on serum glucose concentrations. *db/db* mice (■, n = 5–6) were injected intraperitoneally with 50 mg/kg of indicated oligonucleotides for 4 weeks. Lean littermates (□, n = 5) were dosed with saline or 100 mg/kg of AS1. Statistics were performed using ANOVA, followed by Bonferroni-Dunn. Compared with saline-treated, *P < 0.005. *C*: Insulin tolerance test in PTEN antisense–treated mice. Mice were treated once a week for 3 weeks with saline (♦) or 50 mg/kg AS1 (●) (n = 5). Lean controls (x) were untreated. Results are expressed as the mean ±SE percentage of the glucose concentration at time 0. Statistical analysis was done using ANOVA repeated measures followed by Bonferroni-Dunn. Compared with saline-treated, *P < 0.05.

that PTEN mRNA and protein levels were reduced to the same extent as that of *db/db* mice.

To determine whether inhibition of PTEN expression had an effect on insulin sensitivity, an insulin tolerance test was performed in *db/db* mice treated once a week for 3 weeks with saline or AS1 (Fig. 3*C*). PTEN AS1 significantly increased sensitivity to insulin; the relative blood glucose concentrations in the AS1-treated mice were significantly lower at all time points after insulin injection compared with those in saline-treated animals. Also, inhibition of PTEN expression did not appear to cause hypoglycemia, in that glucose levels in mice remained normoglycemic in PTEN AS1–treated *db/db* and lean mice fasted for 16 h (data not shown).

Inhibition of PTEN expression also lowered serum triglyceride and cholesterol concentrations in *db/db* mice in a dose-dependent manner (Table1). Lipid concentrations were unaffected relative to lean littermates at the 50-mg/kg dose of AS1, and the control oligonucleotide had no effect. Treated *db/db* mice gained significantly more weight than saline- and control antisense–treated *db/db* mice, despite the fact that food intake was similar in all groups. However, *ob/ob* mice did not gain weight relative to saline-treated animals during their 4-week antisense treatment (see below). No significant changes in body composition (i.e., lean versus fat body mass) were observed in any mice treated with PTEN ASOs (data not shown).

The effect of inhibiting PTEN expression on hyperinsulinemia was investigated in *ob/ob* mice, which have higher circulating levels of insulin and are less hyperglycemic than *db/db* mice. Male *ob/ob* mice were injected with 50 or 20 mg/kg of AS1 on day 0 and then with either 20 or 10 mg/kg a week thereafter for 3 weeks, a dosing schedule designed to attain more moderate steady-state levels of oligonucleotide in liver. At the end of 4 weeks, PTEN

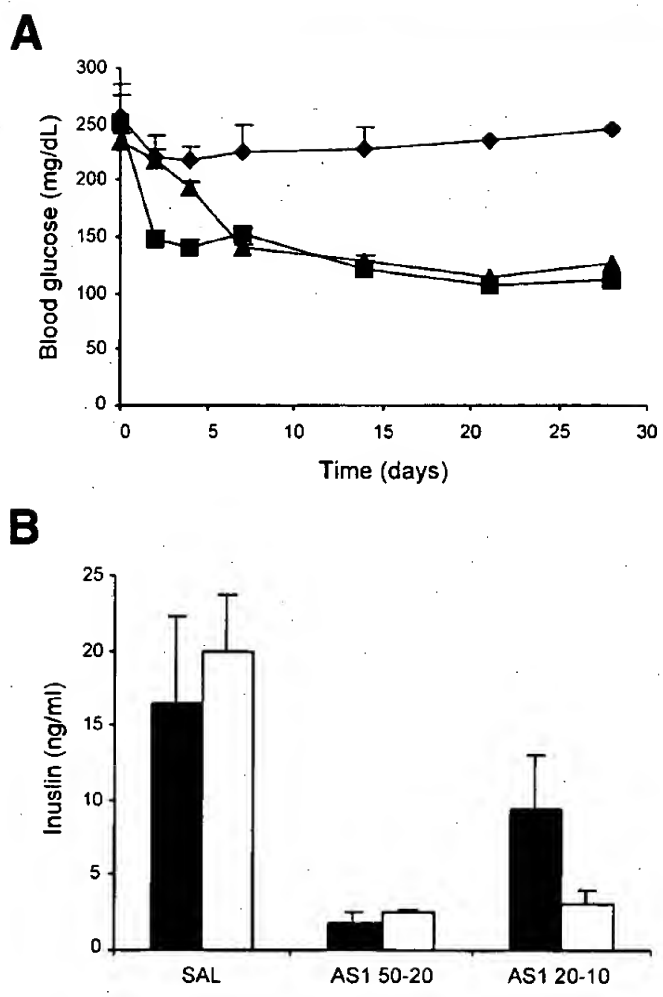

FIG. 4. Reduction of blood glucose (*A*) and serum insulin (*B*) in *ob/ob* mice treated with PTEN antisense. *A*: *Ob/ob* mice were injected intraperitoneally once a week with saline (♦) or 50 mg/kg AS1 on day 0, followed by 20 mg/kg once a week thereafter (■), or 20 mg/kg AS1 on day 0, followed by 10 mg/kg once a week thereafter (▲). Values are expressed as means ± SE (n = 8). *B*: *ob/ob* mice treated as described above were fasted for 4 h before measuring serum insulin after 2 weeks (■) and 4 weeks (□) of dosing. Values are expressed as the means ± SE (n = 6).

mRNA levels were reduced by 71% in the higher-dose group and by 49% in the lower-dose group (data not shown) relative to saline-treated animals, and blood glucose concentrations were normalized by 2 weeks in both dose groups (Fig. 4*A*). Serum insulin levels were reduced by 90% at 2 weeks in the higher-dose group and by 84% at 4 weeks in the lower-dose group (Fig. 4*B*).

Effect of inhibiting PTEN expression on Akt phosphorylation in diabetic and lean mice. If the effects of PTEN inhibition on glucose and insulin levels in diabetic mice are caused by an activation of PI3K signaling, evidence of a biochemical improvement in insulin signaling downstream of PI3K should be detectable. Because Akt activation is dependent on the products of PI3K, we reasoned that decreasing PTEN expression would result in increased levels of Akt phosphorylation in response to insulin in diabetic animals. To test this, *ob/ob* mice and their lean littermates were treated with either saline, AS1, or MIS at 50 mg/kg once a week for 2 weeks, and PTEN and phospho-Akt protein levels were determined in liver. As can be seen in Fig. 5*A*, PTEN protein expression was reduced by ~90% in livers from both lean and *ob/ob* mice treated with AS1, relative to saline-treated mice. In lean mice, neither the basal levels of Akt phosphorylation nor the sixfold increase in Akt phosphorylation in response to a bolus insulin injection were affected by PTEN antisense treatment (Fig. 5*B*). As has been previously reported in diabetic rats (29), no increase in Akt phosphorylation in response to insulin was observed in control-treated *ob/ob* mice. However, PTEN AS1 treatment appeared to restore Akt phosphorylation in response to insulin in *ob/ob* mice (Fig. 5*C*). However, PTEN AS1 treatment did not appear to affect basal levels (non–insulin-stimulated) of phosphorylated Akt in *ob/ob* mice.

DISCUSSION

The molecular defects that cause insulin resistance and hyperglycemia in type 2 diabetes have not been well defined. Impaired insulin receptor function leading to reduced activation of PI3K could be a cause of insulin resistance, or the primary defect may lie further downstream in the PI3K pathway. The results presented here indicate that PTEN, a tumor suppressor with phosphoinositide 3′-phosphatase activity, may play a role in glucose metabolism in vivo by negatively regulating insulin signaling.

Several lines of evidence indicate that PI3K activation and the subsequent rise in PIP3 concentrations are necessary for many of the metabolic responses to insulin, including Akt activation, glucose transport, and glycogen and lipid synthesis. PTEN is capable of dephosphorylating PIP3 (12), and cells in which PTEN activity has been inhibited have elevated PIP3 concentrations and higher levels of Akt phosphorylation (16). Thus, it seems logical that PTEN can regulate insulin signaling through the PI3K pathway. Indeed, Nakashima et al. (19) recently demonstrated that overexpression of PTEN in 3T3-L1 cells inhibits glucose uptake and GLUT4 translocation in vitro, whereas microinjection of a PTEN antibody increased basal and insulin-stimulated GLUT4 translocation also in vitro. Our results demonstrating that antisense-mediated reduction of PTEN expression increased insulin-dependent Akt phosphorylation in vitro also supports the conclusion that PTEN may negatively regulate insulin signaling in cultured cells.

Moreover, using our antisense approach, we were able to determine the effect of inhibiting PTEN expression in animals, and our results suggest that PTEN plays an important role in glucose homeostasis in vivo as well. We have shown that systemic administration of PTEN antisense reduced PTEN mRNA and protein expression in a dose-dependent manner in mouse liver but had no effect on the levels of the phosphatases PTP1B and SHIP2. Similar results have recently been obtained using a Fas ASO, which reduced Fas mRNA and protein expression in hepatocytes by up to 90% after systemic injection in mice (25). We also found that systemically administered PTEN oligonucleotides are capable of reducing target expression in fat, but not muscle, and that PTEN mRNA and protein levels are much less abundant in muscle than in fat and liver. Taken together, these results indicate that the effects of the antisense were indeed specific for PTEN and suggest that the reduction of PTEN expression primarily in

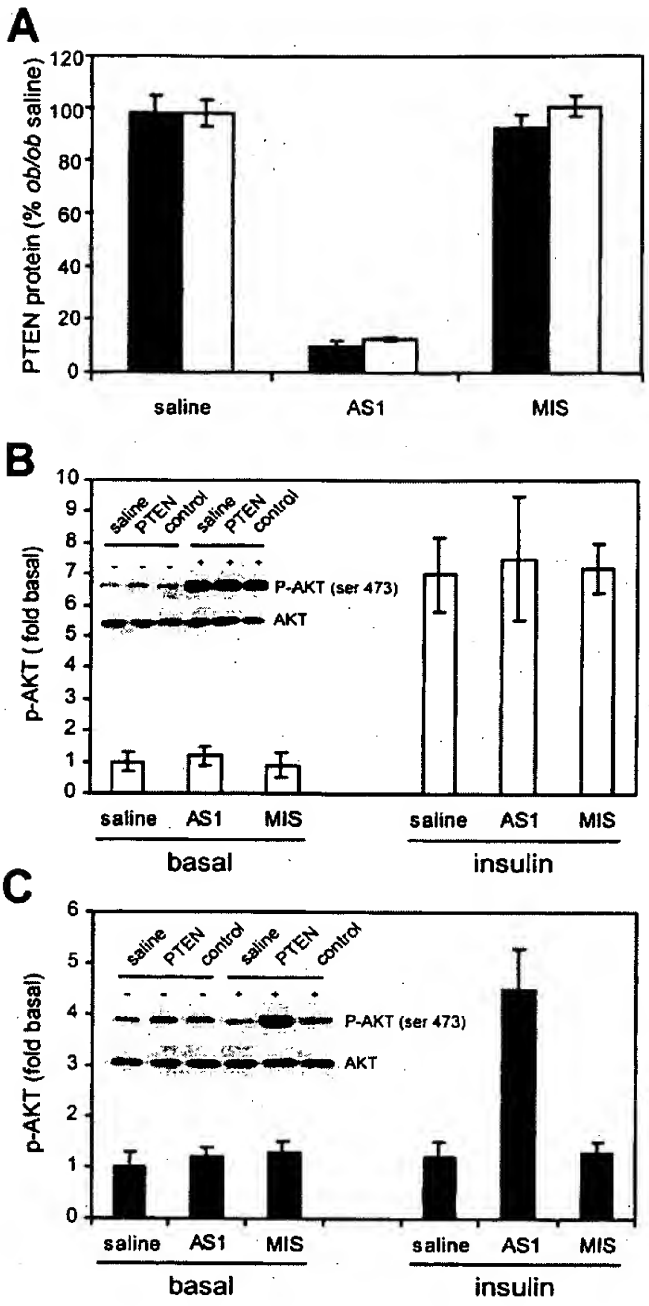

FIG. 5. Reduction of PTEN expression in *ob/ob* and lean mice and increased Akt phosphorylation in *ob/ob* mice treated with PTEN antisense. *A*: Reduction of PTEN protein levels in both *ob/ob* (■) and lean (□) mouse livers after PTEN antisense treatment. Mice (n = 4 per group) were injected for 2 weeks with saline or 50 mg/kg ASI or MIS oligonucleotides, and liver lysates were immunoblotted with antibodies to PTEN and G3DPH. Data are expressed as the mean percentage of normalized PTEN protein levels in saline-treated *ob/ob* mice ±SD. *B* and *C*: Phospho-Akt levels in livers of lean and *ob/ob* mice without (basal) or 5 min after intraperitoneal injection of 2 units/kg of insulin. Immunoblots of proteins were sequentially reacted with antibodies to phospho-Akt and Akt. Data are expressed as the fold increase in phospho-Akt intensity compared with basal levels in saline-treated mice relative to Akt protein levels. Graphs represent the means ± SD. Representative gels of pooled samples (n = 3) from each group are also shown for lean and *ob/ob* mice.

liver, with some possible contribution in fat, was sufficient to produce the observed metabolic changes in diabetic mice. Improved insulin sensitivity in liver of diabetic mice would be expected to reduce hepatic glucose production and thereby reverse hyperglycemia.

Antisense-mediated inhibition of PTEN expression normalized glucose concentration in both *db/db* and *ob/ob* mice, improved insulin sensitivity in *db/db* mice, and lowered insulin concentrations dramatically in *ob/ob* mice. The fact that inhibition of PTEN expression reversed hyperglycemia and reduced insulin resistance in diabetic mice, without affecting glucose levels in lean mice, suggests that the reduction in PTEN expression compensated for a defect in the PI3K pathway in diabetic mice. Alternatively, it is possible that inhibition of PTEN in diabetic mice may somehow compensate for defects in other pathways that are unrelated to PI3K but may contribute to insulin resistance in these animals. No detectable difference in PTEN mRNA or protein levels in lean versus *db/db* mice was observed, so it does not appear that an increase in PTEN expression levels is the primary defect in these mice. It has previously been demonstrated that IRS-associated PI3K activity is decreased significantly in *ob/ob* mouse liver (30) and that Akt/PKB activity is reduced in liver and muscle from diabetic rats and humans (29). We have demonstrated that inhibition of PTEN expression in vivo restores insulin-stimulated Akt phosphorylation in *ob/ob* diabetic mice to a level comparable with that in lean mice. We have also observed significant increases in liver Akt phosphorylation in *db/db* mice treated with PTEN antisense (data not shown). Thus, it seems logical that a reduction in PTEN expression after antisense treatment resulted in increased PI3K activity by increasing the half-life and/or effective concentration of PIP3 produced during insulin activation. This logic is consistent with the putative role of PTEN in PI3K signaling, as well as with the results of other investigators who have employed different approaches for suppressing PTEN activity in vitro (11–14,17–19). Nevertheless, we cannot rule out the possibility that the effects of PTEN inhibition on Akt phosphorylation and insulin sensitivity that we have observed in vitro and in vivo may not be directly related to increased PI3K activity and increased PIP3 levels because neither of these end points were measured directly. Although PTEN antisense treatment had no effect on insulin-dependent Akt phosphorylation or on circulating glucose levels in lean mice, serum insulin levels were decreased by 50%, suggesting that PTEN inhibition may increase insulin sensitivity in lean mice as well. Interestingly, a recent report about a Cowden's disease patient with a heterozygous PTEN mutation indicated improved insulin sensitivity, as measured by glucose clearance and hyperinsulemic-euglycemic clamp (31). However, although several groups have demonstrated an increased incidence of tumors in PTEN heterozygous mice, no changes in blood glucose concentrations have been reported. This finding may not be surprising in view of the fact that in our studies, reducing PTEN expression by 90% had no significant effect on glucose levels in lean mice.

In conclusion, our results demonstrate that suppression of PTEN expression produces a marked improvement in blood glucose concentrations and insulin sensitivity in diabetic mice and suggest that pharmacological inhibition of negative regulators of the PI3K pathway may represent

a therapeutic approach for the treatment of type 2 diabetes.

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